BIKEN JOURNAL 5, 99-108 (1962)

The Appearance of Intranuclear Inclusion Bodies Induced by Herpes Simplex Virus in FL Cells

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(Received for publication, June 20, 1962)

SUMMARY

Using the -GCr Miyama strain of herpes simplex virus the appearance of the inclusion bodies in FL cells was investigated. The number of inclusions detected between 8 and 9 hours after virus inoculation approximately fitted the Poisson's distribution assuming one hit. From the fact that the number of infectious virus particles expected from the count of inclusion bearing cells coincides with the viral titer counted by the microplaque method, it seems that one infectious virus particle is sufficient to infect an interphase cell and to cause the formation of an intranuclear inclusion in it. Inclusion body-like structures were often seen in the nuclei of mitotic cells, although their nature is unknown.

INTRODUCTION

The developmental features of intranuclear inclusion bodies of herpes simplex virus were reported by Crouse *et al.* (1950), using chorioallantoic membranes of developing eggs and studies were also made by McNair-Scott *et al.* (1953) using rabbit corneal cells *in vitro*.

In an earlier report we described the characteristic pattern of incorporation of H^3-thymidine into the nuclei of herpes virus-infected FL cells seen by autoradiography. (Nii *et al.*, 1960; Nii *et al.*, 1961a) To study the precise developmental cycle of herpetic inclusion bodies, however, it seemed necessary to make a statistical study of the cytological changes. Such studies have been made with other viruses, *e. g.* ectromelia virus and vaccinia virus. (Kamahora *et al.*, 1959; Cairns, 1960)

A preliminary study reported by Ben-Porat *et al.* (1961) showed that at an early stage the number of inclusion bodies induced by pseudorabies virus fitted a Poisson distribution as a function of the multiplicity at infection.

This report describes one aspect of the developmental cycle of herpetic inclusion bodies on monolayer cultures.

MATERIALS AND METHODS

1. *Virus*

-GCr Miyama strain was used. (Nii *et al.*, 1961b) The virus was obtained from the culture

fluid of infected FL cells and the supernatant after centrifugation contained about 10^8 $TCID_{50}$/ml.

2. *Cells*

FL cells were prepared on 10×40 mm coverslips in square tubes. Two or three days after planting, a monolayer containing $5 \times 10^5 \sim 1 \times 10^6$ cells was obtained. This was used for the experiments.

3. *Cell counting*

The number of cells on coverslips was counted in the following way. Before virus inoculation two or three square tubes were taken out of the incubator and the coverslips were removed from them. Each coverslip was put into a centrifugation tube, containing 0.1M citric acid and incubated at 37°C for 1 hour. The cells were detached from the coverslip by spraying the slip vigorously with the liquid. After the liquid had been pippetted back and forth, the cell nuclei could be sedimented by centrifugation at 600 g for 10 minutes. They were resuspended in 2 ml of 0.1M citric acid containing 0.01 per cent gentian violet and 0.25 per cent methylcellulose. Cells were counted in a haemocytometer.

4. *Virus infectivity titration to measure the virus multiplicity of infection*

Virus titration was performed by the microplaque method. (Farnham, 1958) Half log or log serial dilutions of the original viral sample were made with L-E solution containing 10 per cent bovine serum. One ml of each dilution was inoculated into two square tubes each containing a coverslip. After the same adsorption period used for measuring the number of inclusion-bearing cells, the inocula were removed and the coverslips were washed five times with warm Hanks' BSS. Then maintenance medium was introduced. Forty eight hours after inoculation, the coverslips were fixed and stained with hematoxylin. Microplaques were counted under a microscope at a magnification of 100. Forty-eight hours after inoculation was found to be a suitable time for counting microplaques in experiments using the Miyama strain of herpes simplex virus. (Nii *et al.*, 1961b)

5. *Experimental procedures*

Usually experiments were carried out as follows. Serial two or three fold dilutions of the original virus sample were made with L-E solution (Earle's salt solution containg 0.5 per cent Lactalbumin hydrolyzate) containing 10 per cent bovine serum. The culture fluids of square tubes were pipetted off or decanted and one ml of aliquots of each virus dilution were introduced into a series of replicate cultures. After an adsorption period of half or one hour at 37°C, the inocula were discarded and the cultures were washed five times with 2 ml of warm Hanks' balanced salt solution. Then warm maintenance media (L-E solution containing 5 per cent salt solution) was introduced onto the cultures.

At appropriate intervals, one or two square tubes of each virus dilution were taken out of the incubator and the culture cells were fixed with Bouin's solution. Thus a sequential study of infection with different virus dilutions was possible. Cells were stained with hematoxylin and eosin and the number of the inclusion body bearing cells was measured by examming one thousand infected cells.

RESULTS

1. *The appearance of the inclusion bodies*

A series of variable cytological changes took place in infected cells as infection proceeded. However the most characteristic feature of viral infection in the cells was the formation of eosinophilic inclusions. Therefore a cell bearing a definite

spot of eosinophilic material in the nucleus was regarded as an infected cell. The herpetic inclusion itself changes in shape and size from its first appearance. At first it looks pinky purple in the chromatin. At the final stage it appears as a typical inclusion body with a halo or as a full inclusion body. Whatever stage it was seen at it was taken as an inclusion in the count.

In experiment No. 1 the adsorption period was one hour. As shown in Fig. 1, in cultures inoculated with the highest virus multiplicity, *i. e.* the original viral

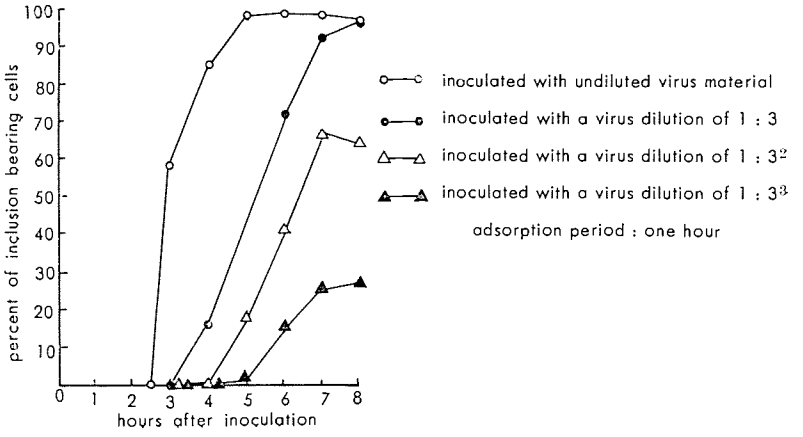

Fig. 1. Inclusion Development (A)

sample, there was very rapid appearance of inclusions, which appeared as early as 3 hours after inoculation, and at the 5th hour almost all the cells were infected. In cultures infected with a lower virus multiplicity the appearance of inclusions was delayed. Therefore within the first 5 hours after virus inoculation, the number of inclusions did not fit a one hit Poisson's distribution curve. Such a tendency for inclusions to appear earlier with higher doses of viral multiplicity was also shown in subsequent experiments, in which a half hour adsorption period was chosen. This fact is probably not explainable merely as due to the rapid adsorption of virus in cultures inoculated with a higher multiplicity of virus. At an early stage of infection using a high viral dose, the nuclei were filled with somewhat large mass of eosinophilic materials surrounded by a narrow band of chromatin material lining the nuclear membrane and abnormal nucleolei associated with condensed chromatin were sometimes seen in the nuclei. So the eosinophilic material often took the form of a ring in the nucleus (Figs. 2 and 3) which may correspond to the ring shaped incorporation of H^3-thymidine. (Nii *et al.*, 1960; Nii *et al.*, 1961a) On the other hand in cultures infected with a lower multiplicity of virus, the inclusion sometimes appeared to occupy part of the nucleus, as shown in Figs. 4 and 5. Condensation of the chromatin surrounding an inclusion was

more or less remarkable. Thus the appearance of inclusions seemed to vary with the virus multiplicity used for induction, but at later stages of infection, *e. g.* from about 6 hours after inoculation, this difference became indistinct. Therefore there may be some analogy between this virus and the pox viruses. In both cases the centers of virus synthesis may be initiated according to the number of input viruses.

Even in cultures inoculated with a lower multiplicity of virus, the number of

Table 1. Relationship between Virus Multiplicity and Number of Inclusion Bodies (A)

Exp. 1 Samples were taken five and o half hours after virus inoculation

virus dilution	$\times 3^0$	$\times 3^1$	$\times 3^2$	$\times 3^3$	$\times 3^4$
percent of inclusion bearing cells*	92.7 93.7	59.4 62.7	19.9 16.4	7.9 9.3	0.8 0.6
average value in parenthesis	(93.2)	(61.1)	(18.2)	(8.6)	(0.7)
expected virus multiplicity△	2.69	0.94	0.20	0.09	0.007
expected multiplicity × dilution	2.69	2.82	1.8	2.43	0.57

Exp. 2 Samples were taken 6 hours after virus inoculation

virus dilution	$\times 3^0$	$\times 3^1$	$\times 3^2$	$\times 3^3$	$\times 3^4$	$\times 3^5$
percent of inclusion bearing cells*	98.5	95.8	91.0 84.6	51.2 50.3	17.7 17.3	3.1 4.6
average value in parenthesis			(87.8)	(50.8)	(17.5)	(3.9)
expected virus multiplicity△	4.20	3.17	2.10	0.71	0.19	0.04
expected multiplicity × dilution	4.20+	9.51+	18.9	19.2	15.4	9.72

Exp. 3 Samples were taken 7 hours after virus inoculation

virus dilution	$\times 3^0$	$\times 3^1$	$\times 3^2$	$\times 3^3$	$\times 3^4$	$\times 3^5$
percent of inclusion bearing cells*	96.5 97.8	93.6 92.5 91.2	53.5 52.7 61.4	24.5 27.0 27.7 27.7	12.2 11.4	4.0 4.0 2.2
average value in parenthesis	(97.2)	(92.4)	(55.9)	(26.7)	(11.8)	(3.4)
expected virus multiplicity△	3.57	2.58	0.82	0.31	0.13	0.035
expected multiplicity × dilution	3.57+	7.74	7.37	8.40	10.2	8.51

* percent of inclusions calculated from counts on 1000 cells on a covership.

△ Calculated by Poisson's law assuming one hit event.

\+ Low values at lower dilutions were chiefly due to counts of mitotic cells.

inclusion-bearing cells increased until 7-8 hours after inoculation when it reached nearly its maximum. From the 7th hour, the number of inclusions approximately fitted a Poisson distribution curve. (Table 1) Subsequently the curves showed a plateau which suggested the first cycle of inclusion body development. (Figs. 1 and 6) Between 8 and 9 hours after infection, the number of inclusions also fitted the Poisson's law well. (Table 2)

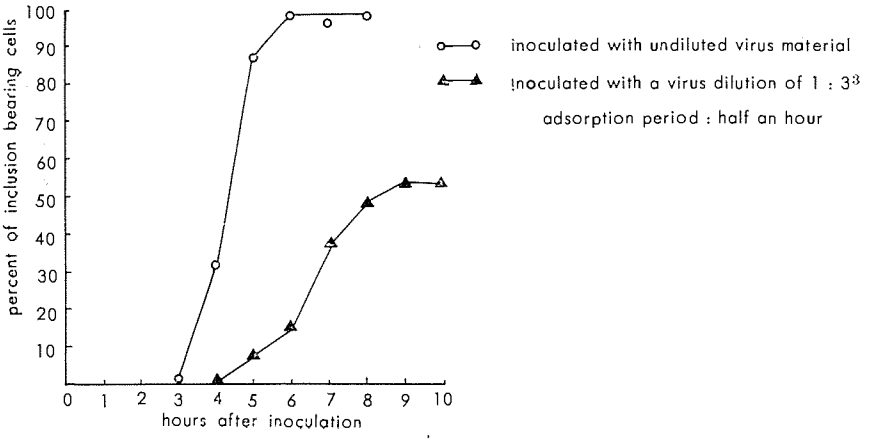

Fig. 6. Inclusion Development (B)

Table 2. Relationship between Virus Multiplicity and Number of Inclusion Bodies (B)

Experiment	virus dilution	$\times 3^0$	$\times 3^1$	$\times 3^2$	$\times 3^3$	$\times 3^4$
Exp. a	percent of inclusion bearing cells*	98.0	95.3	65.5	27.0	
	expected virus multiplicity△	3.91	3.06	1.07	0.315	
	expected multiplicity × dilution	3.91⁺	9.18⁺	9.63	8.50	
Exp. b	percent of inclusion bearing cells*	99.3	94.1	79.7	38.1	
	expected virus multiplicity△	4.96	2.83	1.59	0.48	
	expected multiplicity × dilution	4.96⁺	8.49⁺	14.3	13.0	
Exp. c	percent of inclusion bearing cells*		98.0	81.0	36.7	13.0
	expected virus multiplicity△		3.91	1.66	0.457	0.14
	expected multiplicity × dilution		11.7⁺	14.9	12.2	12.3

Samples were obtained at 8-9 hours after virus inoculation.

* percent of inclusions calculated from counts on 1000 cells on a coverslip.

△ Calculated by Poisson's formula assuming one hit event.

⁺ Low values at lower dilutions seems chiefly due to counts of mitotic cells.

2. *Relationship between the inclusion forming units and the plaque forming units*

The inclusion bodies seen at 8-9 hours after infection were assumed to be in the first cycle of development and were counted in the cultures inoculated with each virus dilution and the number of inclusion forming units per coverslip was calculated. The number of plaque forming units was also counted as described in the MATERIALS AND METHODS. The ratio of the former to the latter is shown in Table 3. This ratio was approximately one.

Table 3. Comparison of Numbers of Plaque Forming Units and Inclusion Forming Units

Exp. No.	P. F. U. number	I. F. U. number	P. F. U. / I. F. U.
Exp. I	9.0×10^5	8.4×10^5	1.1
Exp. II	1.5×10^7	1.1×10^7	1.4
Exp. III	1.0×10^7	1.0×10^7	1.0

3. *Possibility of recycling*

As mentioned above, the number of inclusion bearing cells detected at 8-9 hours after virus inoculation was recognized to be due to one cycle of virus infection. To find whether the inclusion percentage fits a Poisson distribution curve only at this period it is necessary to know the time of recycling.

Experiments were made on the developmental cycle of inclusions for longer periods. As shown in Figs. 7 and 8 the developmental curves of inclusions had a plateau at between 8 and 10 hours after virus inoculation and this was more pronounced at higher virus dilutions. The number of inclusions began to rise again

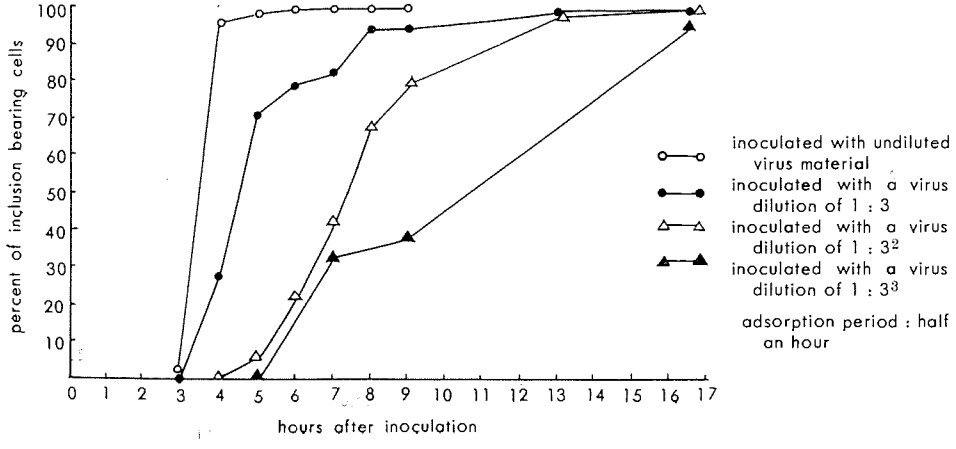

Fig. 7. Inclusion Development (C)

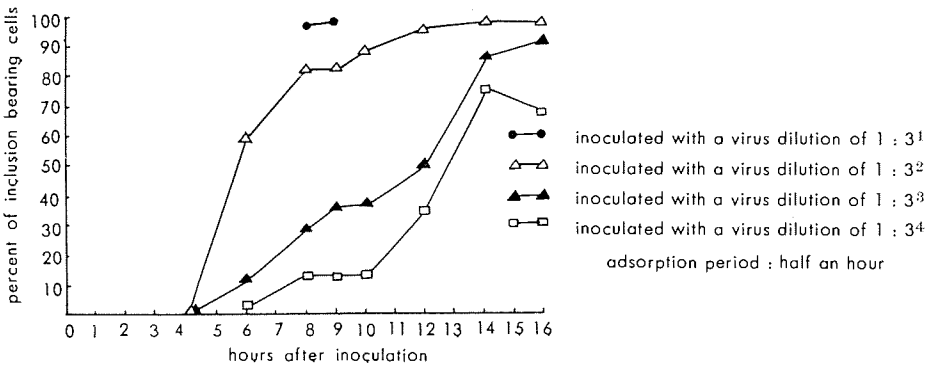

Fig. 8. Inclusion Development (D)

between 10 and 12 hours after inoculation, as shown in Fig. 8, and this was more marked in cultures infected with lower multiplicities of virus. This result suggests the recyling of infection.

This conclusion is supported by growth curve experiments. As already reported, the viral titer of the -GCr Miyama strain of herpes simplex virus rose in culture fluids within 8 hours after infection. (Nii *et al.*, 1961b) In this series of experiments, inclusions started to appear in cultures infected with a high virus input 3 hours after inoculation. In cultures inoculated with a low virus multiplicity uninfected cells adjacent to infected cells may be exposed to multiple infection. Therefore the second rise in the number of inclusion bodies seen 11 hours after virus inoculation indicates recycling.

4. *The effect of virus infection on mitotic cells*

Table 4 shows the effect of virus infection on mitotic cells. In this experiment

Table 4. Effect of Virus Infection on Mitotic Activity

		hours after virus inoculation							
		3	4	5	6	7	8	9	10
percent of mitotic cells inoculated with a virus dilution of	1 : 3^0	0,9	1,4	0,8	1,3	1,3	1,2		
	1 : 3^1		2,4	1,6	1,8	2,7	2,9		
	1 : 3^2		2,7	2,5	3,4	3,3	2,5	2,6	1,8
	1 : 3^3		2,7	3,4	4,1	4,3	3,9	2,8	3,2

The adsorbed virus multiplicity of undiluted sample was 31 PFU per cell.
The percent of mitotic cells were calculated from counts on 1000 cells.

virus samples were prepared by serial threefold dilution of the sample. The origi-

nal virus multiplicity was 31 PFU per cell. A half hour adsorption period was chosen. In infected cells inoculated with a low virus dilution there was a stronger inhibitory effect on mitosis than with high virus dilutions.

Morphologically abnormal mitotic figures were always seen and inclusion body-like structures were often seen in them. In control cells, such structures were never seen. (Figs. 9, 10, 11 and 12)

DISCUSSION

Kamahora *et al.* (1959) studied the "B" type inclusion bodies induced by ectromelia virus in Ehrlich ascites tumor cells from the statiscal point of view and concluded that one "B" body was formed by one infectious unit of virus. Essentially the same phenomenon was reported by Cairns for vaccinia virus infection, using autoradiography. (Cairns, 1960)

In earlier reports we described the intranuclear changes in herpes-infected cells labeled with H^3-thymidine using an autoradiographic technique and showed that at an early stage of infection concentrated grains appeared as islands or rings inside the nuclear membrane. (Nii *et al.*, 1960; Nii *et al.*, 1961a) It is interesting to find the correlation between the counts of grains in infected cells and the developmental features of the herpetic inclusion bodies. Therefore it was most important to recognize the earliest definite sign of infection with herpes simplex virus. The development of intranuclear inclusions was described by Scott *et al.* (1953) in rabbit corneal cells grown in tissue culture. They noted that the first fairly definite evidence of inclusion material was seen about 6 hours after infection. (Scott, 1961)

Our experiments showed that the inclusions detected between 8 and 9 hours after infection approximately fitted the Poisson's distribution and in this period the number of inclusion forming units and of plaque forming units were about equal. So it seems that one inclusion is induced by one infectious unit of herpes virus. However the appearance of the inclusion material is very much influenced by the virus multiplicity. This is apparently not only due to the earlier adsorption of virus in cultures inoculated with higher multiplicities of virus, but also to the multiple initiation of infective centers in the nuclei. In a cell infected with a high virus multiplicity, the site of virus reproduction spreads diffusely through the whole nucleus earlier than in a cell infected with only infective virus and this would cause the earlier appearance of intranuclear inclusion bodies. The morphological difference in the early inclusions caused by different viral doses was reported in this paper. However, this is noticed only in the early stages of inclusion body development.

As inclusion bearing cells were not counted in interphase cells only, the percentage of cells with inclusions did not reach one hundred even with high multiplicities of virus. A very small number of abnormal mitotic cells were always en-

countered. The effect of virus infection on mitotic cells must be studied. Mitotic cells in infected cultures often contain inclusion-body like structures and these closely resemble the inclusion bodies formed in mitotic cells infected with canine hepatitis virus, as described by Fastier (1959). However, we did not count these as inclusions, because the nature of these structures has not been studied by immnofluorescent and cytochemical methods.

The number of inclusions in interphase cells in cultures infected with low dilutions of virus was often estimated to be a little lower than expected and this may be due to the presence of cells in the cultures in a poor physiological state.

Experiments on the one cycle development of inclusions are now under way with other variants of the Miyama strain, *i. e.* variants +GC and −GCf. Variant +GC produces inclusion bodies as well as syncytia in infected cells. (Nii *et al.*, 1961b; Nii *et al.*, 1961c) In an earlier report the mass effect of +GC virus was thought to be the cause of syncytial formation. The inclusions induced by this variant between 8 and 9 hours after infection also fitted the Poisson's distribution, while at this stage after infection many polynucleated cells could already be seen even in cultures inoculated with a low multiplicity of virus. The latter phenomenon indicates the effect of virus released from the first infected cells and suggests an early sign of the initiation of a subsequent cycle. The second rise in the number of inclusion bodies, *i. e.* the definite sign of the second cycle was recognized from the 12th hour after virus inoculation.

In the host virus interaction between FL cells and the Miyama strain of herpes simplex virus, one infectious virus is found to be sufficient to induce an intranuclear change in one interphase cell. In the 50 per cent endpoint titration method in tube cultures ($TCID_{50}$), L cells always showed a little lower infectivity titer than FL cells. Therefore it would be interesting to study the appearance of inclusions in L cells and to compare the numbers of the inclusion forming units in this cell line with those in FL cells. This problem is now in progress and results will be published later.

ADDENDUM

When this work with the −GCr strain of Miyama virus was almost complete, similar findings were reported by Wildy *et al.* (1961). Their conclusions were the same as ours with a few difference. Our data indicated that the number of inclusion bearing cells fits the Poisson's distribution between 8 and 9 hours after infection, while their studies using the HFEM strain and HeLa cells indicated that counts at the 14th hour after infection were in good accordance with virus multiplicity. Although details have not been published, the difference in the time suitable for counting inclusions and microplaques seems to be due to the different growth kinetics of the two virus strains.

ACKNOWLEDGEMENTS

We would like to express our gratitude to Dr. Scott, T. F. McN. for his interest to our work.

We are also indebted to Miss Hisako Senno for excellent technical assistance.

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EXPLANATION OF FIGURES

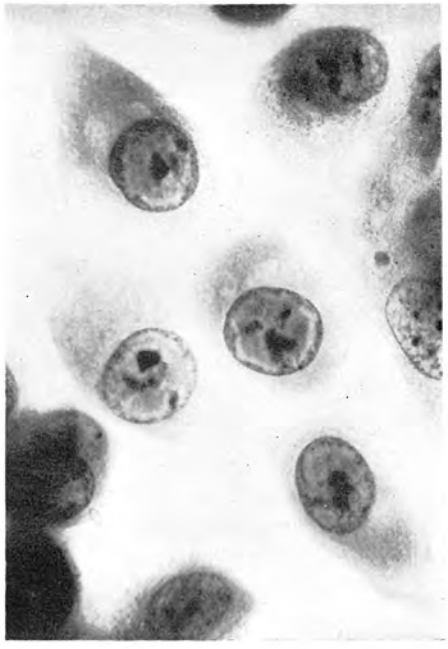
Fig. 2, 3. Intranuclear changes in FL cells infected with the —GCr Miyama strain of herpes simplex. Virus multiplicity 13.5. Four hours after inoculation all nuclei are filled with eosinophilic material surrounded by a narrow chromatin margin lining the nuclear membrane and abnormal nucleolei associated with condensed chromatin are seen in the middle of the nuclei.
× 1800

Fig. 4, 5. Intranuclear changes in FL cells infected with the —GCr Miyama strain of herpes simplex virus. Virus multiplicity 0.5. Five hours after inoculation.
In the center of each plate an infected cell is shown. The inclusion occupies part of the nucleus.
× 1800

Fig, 9, 10, 11, 12. Inclusion body like structures detected in mitotic cells in FL cell cultures infected with the —GCr Miyama strain of herpes simplex virus. Seven hours after virus inoculation.
In the center of each plate an abnormal mitotic cell with an inclusion like structure is shown.

Fig. 9 × 3400
Fig. 10 × 2570
Fig. 11, 12 × 1800

Fig. 2.



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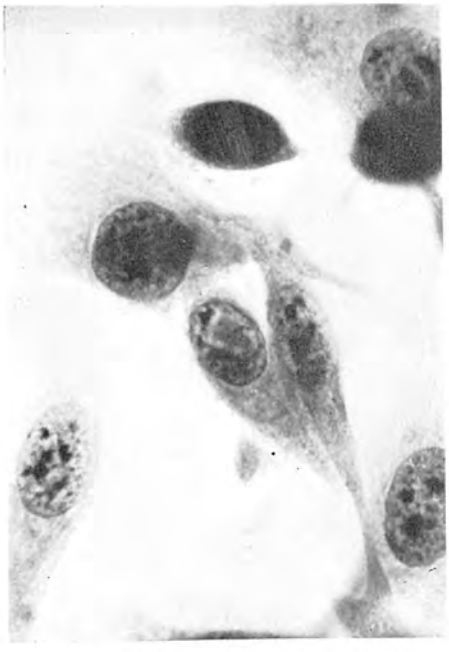

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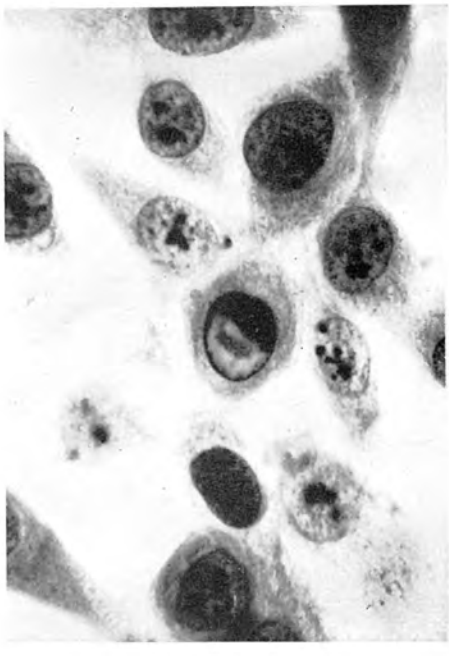

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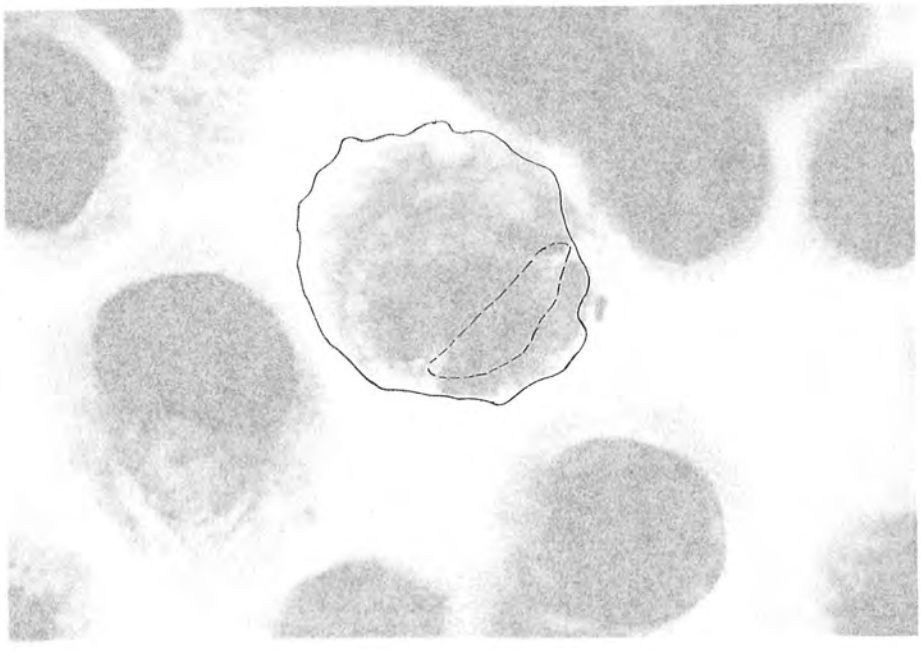


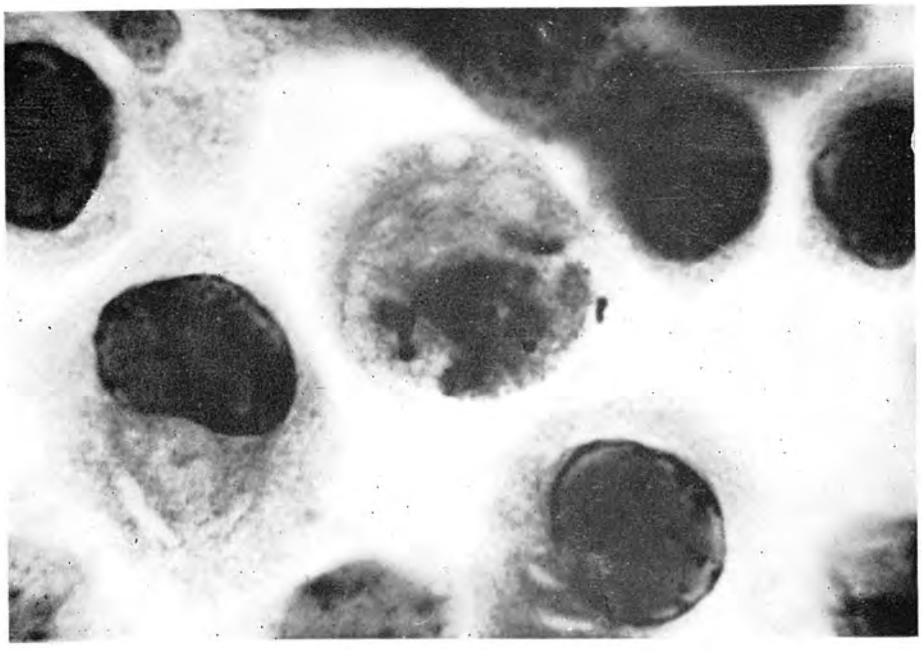


Fig. 9.

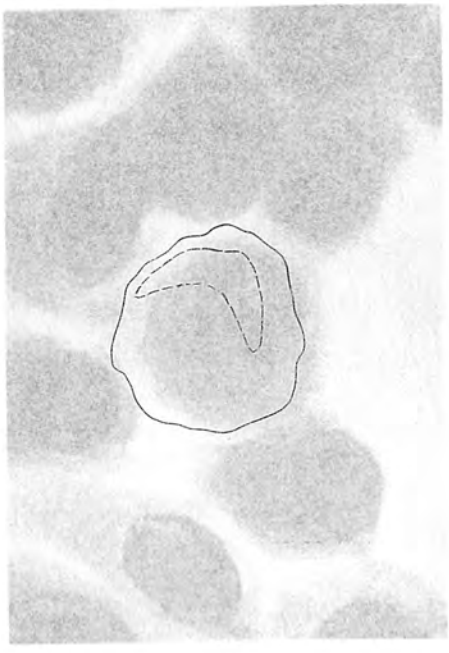
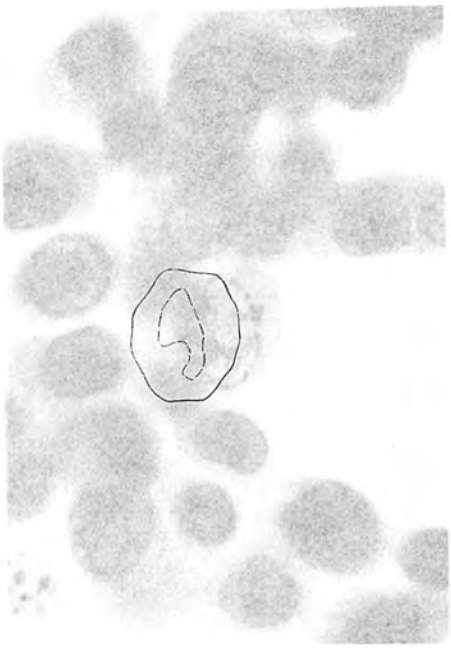


Fig. 10



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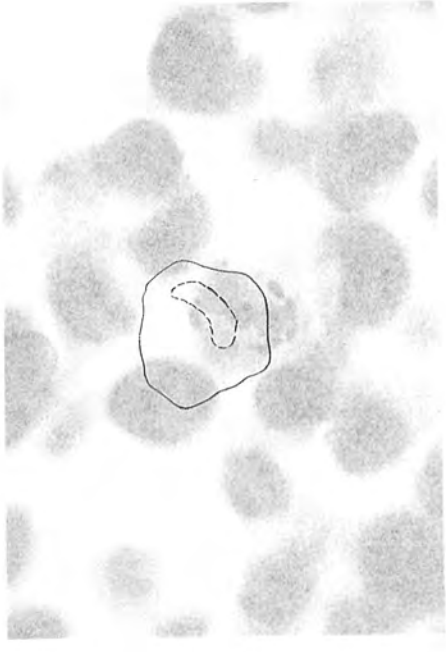

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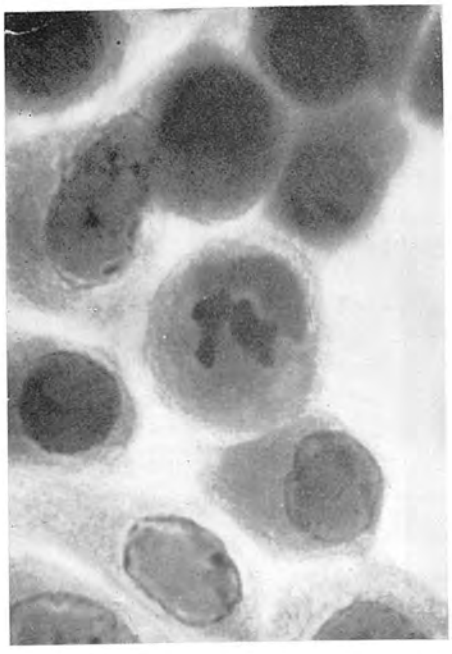

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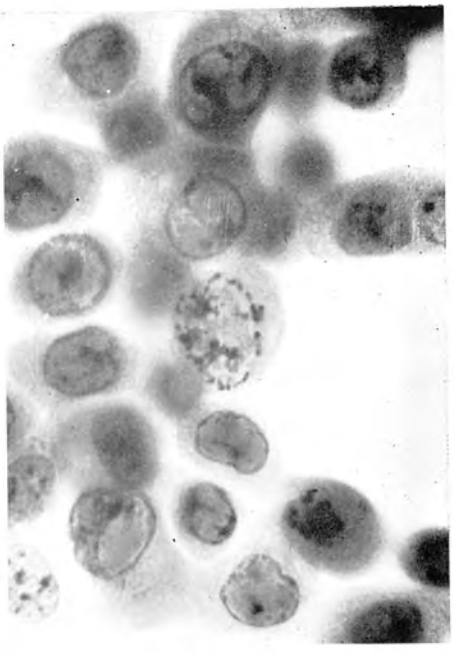

Fig. 11.

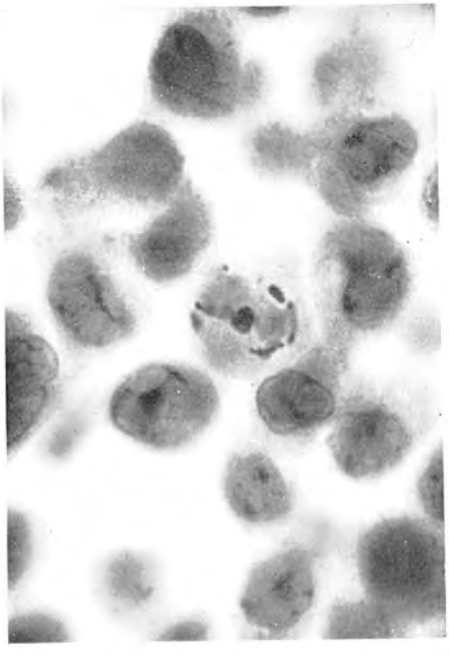

Fig. 12.